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(54) **Fusion proteins containing N-terminal fragments of human serum albumin.**

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EP-A- 0 308 381
EP-A- 0 322 094

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Description

The present invention relates to fusion polypeptides where two individual polypeptides or parts thereof are fused to form a single amino acid chain. Such fusion may erise from the expression of a single continuous coding sequence formed by recombinant DNA techniques.

Fusion polypeptides are known, for example those where e polypeptide which is the ultimately desired product of the process is expressed with an N-terminal "leader sequence" which encourages or allows secretion of the polypeptide from the cell. An example is disclosed in EP-A-116 201 (Chiron).

Human serum albumin (HSA) is a known protein found in tha blood. EP-A-147 198 (Delta Biotechnology) discloses its expression in a transformed host, in this case yeast. Our earlier application EP-A-322 094 discloses N-terminal fragments of HSA, namely those consisting of residues 1-n where n is 369 to 419, which have therapeutic utility. The application elso mentions the possibility of fusing the C-terminal residue of such molecules to other, unnemed, polypeptides.

One aspect of the present invention provides a fusion polypeptide comprising, as at least part of the N-terminal portion thereof, an N-terminel portion of HSA or a variant thereof and, es et least part of the C-terminel portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or e variant thereof then the said polypeptide is (e) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, (c) platelet derived growth factor, or a variant thereof, (d) transforming growth factor, or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alphe-1-antitrypsin or e variant thereof.

The N-terminal portion of HSA is preferably the said 1-n portion, the 1-177 portion (up to and including the cysteine), the 1-200 portion (up to but excluding the cysteine) or a portion intermediete 1-177 and 1-200.

The term "human serum albumin" (HSA) is intended to include (but not necessarily to be restricted to) known or yet-to-be-discovered polymorphic forms of HSA. For example, albumin Naskapi has Lys-372 in place of Glu-372 and pro-albumin Christchurch has an altered pro-sequence. The term "variants" is intended to include (but not necessarily to be restricted to) minor artificial veriations in sequence (such as molecules lacking one or e few residues, having conservative substitutions or minor insertions of residues, or heving minor veriations of amino acid structure). Thus polypeptides which have 80%, preferably 85%, 90%, 95% or 99%, homology with HSA are deemed to be "variants". It is also preferred for such variants to be physiologically equivelent to HSA; that is to say, variants preferably share at leest one pharmacological utility with HSA. Furthermore, any putative variant which is to be used pharmacologically should be non-immunogenic in the animal (especially human) being treated.

Conservative substitutions are those where one or more amino acids are substituted for others having similar properties such that one skilled in the art of polypeptide chemistry would expect at least the secondary structure, and preferably the tertiary structure, of the polypeptide to be substantially unchanged. For example, typical such substitutions include esparagine for glutamine, serine for esparagine and arginine for lysine. Veriants may alternatively, or as well, lack up to ten (preferably only one or two) intermediate amino acid residues (ie not at the terminl of the said N-terminal portion of HSA) in comparison with the corresponding portion of natural HSA; preferably any such omissions occur in the 100 to 369 portion of the molecule (relative to mature HSA itself) (if present). Similarly, up to ten, but preferably only one or two, amino acids may be added, again in the 100 to 369 portion for preference (if present). The term "physiologically functional equivalents" also encompasses larger molecules comprising the said sequence plus a further sequence at the N-terminal (for exemple, pro-HSA, pre-pro-HSA and met-HSA).

Clearly, the said "another polypeptide" in the fusion compounds of the invention cannot be the remaining portion of HSA, since otherwise the whole polypeptide would be HSA, which would not then be a "fusion polypeptide".

Even when the HSA-like portion is not the said 1-n portion of HSA, it is preferred for the non-HSA portion to be one of the said (a) to (h) entities.

The 1 to 368 portion of CD4 represents the first four disulphide-linked immunoglobulin-like domains of the human T lymphocyte CD4 protein, the gene for and amino acid sequence of which are disclosed in D. Smith et al (1987) Science 328, 1704-1707. It is used to combat HIV infections.

The sequence of human platelet-derived growth factor (PDGF) is described in Collins et al (1985) Nature 316, 748-750. Similarly, the sequence of transforming growth factors β (TGF-β) is described in Derynck et al (1985) Nature 316, 701-705. These growth factors ere useful for wound-healing.

A cDNA sequence for the 1-261 portion of Fn was disclosed in EP-A-207 751 (obtained from plasmid pFH6 with endonuclease PvuII). This portion binds fibrin end can be used to direct fused compounds to blood clots.

A cDNA sequence for the 278-578 portion of Fn, which contains e collagen-binding domein, was disclosed by R.J. Owens and F.E. Baralle in 1986 E.M.B.O.J. 5, 2825-2830. This portion will bind to platelets.

The 1-272 portion of von Willebrand's Factor binds and stabilises factor VIII. The sequence is given in Bontham et al, Nucl. Acids Res. 14, 7125-7127.

Variants of alpha-1-antitrypsin include those disclosed by Rosenburg et al (1984) Nature 312, 77-80. In particular, the present invention includes the Pittsburgh veriant (Met^{358} is muteted to Arg) end the veriant where Pro^{357} end Met^{358} are mutated to alanine and arginine respectively. These compounds are useful in the treatment of septic shock end lung disorders.

Variants of the non-HSA portion of the polypeptides of the invention include variations as discussed ebove in relation to the HSA portion, including those with conservative emino acid substitutions, end also homologues from other species.

The fusion polypeptides of the Invention may have N-terminal emino ecids whlch extend beyond the portion corresponding to the N-terminel portion of HSA. For exemple, if the HSA-like portion corresponds to an N-terminal portion of mature HSA, then pre-, pro-, or pre-pro sequences may be added thereto, for example the yeast alpha-factor leader sequence. The fused leader portions of WO 90/01063 may be used. The polypeptide which is fused to the HSA portion may be a naturally-occurring polypeptide, a fragment thereof or a novel polypeptide, including a fusion polypeptide. For example, In Example 3 below, e fragment of fibronectin is fused to the HSA portion via a 4 amino acid linker.

It has been found that the amino terminal portion of the HSA molecule is so structured as to favour particularly efficient translocation and export of the fusion compounds of the invention in eukaryotic cells.

A second aspect of the inventlon provides a transformed host having a nucleotide sequence so erranged es to express e fusion polypeptide as described ebove. By "so errenged", we mean, for example, thet the nucleotide sequence is in correct reading frame with an appropriate RNA polymerase binding slte and translation start sequence and is under the control of a suitable promoter. The promoter may be homologous with or heterologous to the host. Downstream (3') regulatory sequences may be included if desired, as is known. The host is preferably yeast (for example Saccharomyces spp., e.g. S. cerevisiae; Kluyveromyces spp., e.g. K. lactis; Pichia spp.; or Schizosaccharomyces spp., e.g. S. pombe) but may be eny other suitable host such es E. coll, B. subtilis, Aspergillus spp., mammalian cells, plant cells or Insect cells.

A third aspect of the invention provides a process for preparing a fusion polypeptide according to the first espect of the invention by cultivation of a transformed host eccording to the second aspect of the invention, followed by separation of the fusion polypeptide in a useful form.

A fourth aspect of the invention provides therapeutic methods of treatment of the human or other animal body comprising edministration of such e fusion polypeptide.

In the methods of the invention we are particularly concerned to improve the efficiency of secretion of useful therapeutic human proteins from yeast end have conceived the idea of fusing to amino-terminel portions of HSA those proteins which may ordinarily be only inefficiently secreted. One such protein is e potentially veluable wound-healing polypeptide representing amino ecids 585 to 1578 of human fibronectin (referred to herein as Fn 585-1578). As we have described in a separate application (filed simultaneously herewith) this molecule contains cell spreading, chemotectic and chemokinetic activities useful in healing wounds. The fusion polypeptides of the present Invention wherein the C-terminal portion is Fn 585-1578 can be used for wound healing applications es biosynthesised, especially where the hybrid human protein will be topically applied. However, the portion representing amino acids 585 to 1578 of human fibronectin can if desired be recovered from the fusion protein by preceding the first amino acid of the fibronectin portion by amino acids comprising a factor X cleavage site. After isolation of the fusion protein from culture supernatant, the desired molecule is releesed by factor X cleavage end purified by suitable chromatography (e.g. ion-exchange chromatogrephy). Other sites providing for enzymatic or chemical cleavage can be provided, either by appropriate juxtaposition of the N-terminal and C-terminal portions or by the insertion therebetween of an appropriate linker.

Atleast some of the fusion polypeptides of the invention, especially those including the said CD4 and vWF fragments, PDGF and α_1AT, also have an increased half-life in the blood and therefore have advantages and therapeutic utilities themselves, namely the therapeutic utility of the non-HSA portion of the molecule. In the case of α_1AT and others, the compound will normally be administered as a one-off dose or only a few doses over e short period, rather than over a long period, and therefore the compounds are less likely to cause an immune response.

EXAMPLES : SUMMARY

Standard recombinant DNA procedures were as described by Maniatis et al (1982 and recent 2nd edition) unless otherwise stated. Construction and analysis of phage M13 recombinant clones was as described by

Messing (1983) and Sanger et al (1977).

DNA sequences encoding portions of human serum albumin used in the construction of the following molecules ara derived from the plasmids mHOB12 and pDBD2 (EP-A-322 094, Delta Biotechnology Ltd, ralevant portions of which are reproduced below) or by synthesis of oligonucleotides equivalent to parts of this sequence. DNA sequences encoding portions of human fibronectin ara derived from the plasmid pFHDEL1, or by synthesis of oligonucleotides equivalent to parts of this sequence. Plasmid pFHDEL1, which contains the complete human cDNA encoding plasma fibronectin, was obtained by ligation of DNA derived from plasmids pFH6, 16, 54, 154 and 1 (EP-A-207 751; Delta Biotechnology Ltd).

This DNA represents an mRNA variant which does not contain the 'ED' sequence and had an 89-amino ecid variant of the III-CS region (R.J. Owens, A.R. Kornblihtt and F.E. Baralle (1986) Oxford Surveys on Eukaryotic Genes 3 141-160). The map of this vector is disclosed in Fig. 11 and the protein sequence of the mature polypeptlda produced by expression of this cDNA is shown in Fig. 5.

Oligonucleotides were synthesised on an Applied Biosystems 380B oligonucleotide synthesiser according to the manufacturer's recommendations (Applied Biosystems, Warrington, Cheshire, UK).

An expression vector was constructed in which DNA encoding the HSA secretion signal and mature HSA up to and including the 387th amino acid, leucine, fused in frame to DNA encoding a segment of human fibronectin representing amino acids 585 to 1578 inclusiva, was placed downstreem of the hybrid promotar of EP-A-258 067 (Delta Biotechnology), which is a highly efficient galactose-inducible promoter functional in Saccharomyces cerevisiae. The codon for tha 1578th amino acid of human fibronectin was directly followed by a stop codon (TAA) and then the S. cerevisiae phosphoglycerate kinase (PGK) gene transcription terminator. This vector was then Introduced into S. cerevisiae by transformation, wherein it directed tha expression and secretion from the cells of a hybrid molecule representing the N-terminal 387 emino acids of HSA C-terminally fused to amino ecids 585 to 1578 of human fibronectin.

In e second example a similar vector is constructed so as to enable secretion by S. cerevisiae of a hybrid molecule representing the N-terminal 195 amino acids of HSA C-terminally fused to amino acids 585 to 1578 of human fibronectin.

Aspects of the present invention will now be described by way of example end with reference to the eccompanying drawings, in whlch:

Figure 1 (on two sheets) depicts the amino acid sequence currently thought to be the most representative of natural HSA, with (boxed) the alternative C-termini of HSA(1-n);

Figure 2 (on two sheets) depicts the DNA sequence coding for mature HSA, wherein the sequence included in LInker 3 is underlined;

Figure 3 illustretas, diagrammatically, the construction of mHOB16;

Figure 4 illustrates, diagrammatically, the construction of pHOB31;

Figure 5 (on 6 sheets) illustrates the mature protein sequence encoded by tha Fn plasmid pFHDEL1;

Figure 6 illustrates Linker 5, showing the eight constituent oligonucleotides;

Figure 7 shows schematically the construction of plasmid pDBDF2;

Figure 8 shows schematically the construction of plasmid pDBDF5;

Figure 9 shows schematically the construction of plasmid pDBDF9;

Figure 10 shows schematically the construction of plasmid DBDF12, using plasmid pFHDEL1; and

Figure 11 shows a map of plasmid pFHDEL1.

EXAMPLE 1 : HSA 1-387 FUSED TO Fn 585-1578

The following is an account of a preparation of plasmids comprising sequences encoding a portion of HSA, as is disclosed in EP-A-322 094.

The human serum albumin coding sequence used in the construction of the following molecules is derived from the plasmid M13mp19.7 (EP-A-201 239, Delta Biotech- nology Ltd.) or by synthesis of oligonucleotides equivalent to parts of this sequence. Oligonucleotlde were synthesised using phosphoramidite chemistry on an Applied Biosystems 380B oligonucleotide synthesizer according to the manufacturer's recommendations (AB Inc., Warrington, Cheshire, England).

An oligonucleotide was synthesised (Linker A) which represented a part of the known HSA coding sequence (Figure 2) from the PstI site (1235-1240, Figure 2) to the codon for valine 381 wherein that codon was changed from GTG to GTC:

Linker 1

```
                D     P     H     E     C     Y
5'            GAT   CCT   CAT   GAA   TGC   TAT
3' ACGT       CTA   GGA   GTA   CTT   ACG   ATA
                        1247
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```
  A     K     V     F     D     E     F     K
GCC   AAA   GTG   TTC   GAT   GAA   TTT   AAA
CGG   TTT   CAC   AAG   CTA   CTT   AAA   TTT
                1267
P     L     V
CTT   GTC   3'
GGA   CAG   5'
```

Linker 1 was ligated into the vector M13mp19 (Norrander et al, 1983) which had been digested with PstI and HincII and the ligation mixture was used to transfect E.coli strain XL1-Blue (Stratagene Cloning Systems, San Diego, CA). Recombinant clones were identified by their failure to evolve a blue colour on medium containing the chromogenic indicator X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in the present of IPTG (isopropylthio-β-galactoside). DNA sequence analysis of template DNA prepared from bacteriophage particles of recombinant clones identified a molecule with the required DNA sequence, designated mHOB12 (Figure 3).

M13mp19.7 consists of the coding region of mature HSA in M13mp19 (Norrander et al, 1983) such that the codon for the first amino acid of HSA, GAT, overlaps a unique XhoI site thus:

```
                    Asp  Ala
5'      C T C G A G A T G C A      3'
3'      G A G C T C T A C G T      5'
          XhoI
```

(EP-A-210 239). M13mp19.7 was digested with XhoI and made flush-ended by S1-nuclease treatment and was then ligated with the following oligonucleotide (Linker 2):

Linker 2

```
5' T C T T T T A T C C A A G C T T G G A T A A A A G A  3'
3' A G A A A A T A G G T T C G A A C C T A T T T T C T  5'
                      HindIII
```

The ligation mix was then used to transfect E.coli XL1-Blue and template DNA was prepared from several

plaques and then analysed by DNA sequencing to identify a clone, pDBD1 (Figure 4), with the correct sequence.

A 1.1 kb HindIII to PstI fragment representing the 5' end of the HSA coding region and one half of the inserted oligonucleotide linker was isolated from pDBD1 by agarose gel electrophoresis. This fragment was then ligated with double stranded mHOB12 previously digested with HindIII and PstI and the ligation mix was then used to transfect E.coli XL1-Blue. Single stranded template DNA was prepared from meture bacteriophege particles of several plaques. The DNA was made double stranded in vitro by extension from annealed sequencing primer with the Klenow fragment of DNA polymerase I in the presence of deoxynucleoside triphosphates. Restriction enzyme analysis of this DNA permitted the identification of a clone with the correct configuration, mHOB15 (Figure 4).

The following oligonucleotide (Linker 3) represents from the codon for the 382nd amino ecid of mature HSA (glutamate, GAA) to the codon for lysine 389 which is followed by a stop codon (TAA) and a HindIII site and then a BamHI cohesive end:

Linker 3

```
     E   E   P   Q   N   L   I   K   J
5'  GAA GAG CCT CAG AAT TTA ATC AAA TAA GCTTG      3'
3'  CTT CTC GGA GTC TTA AAT TAG TTT ATT CGAACCTAG  5'
```

This was ligated into double stranded mHOB15, previously digested with HincII and BamHI. After ligation, the DNA was digested with HincII to destroy ell non-recombinant molecules end then used to transfect E.coli XL1-Blue. Single stranded DNA was prepared from becteriophage particles of e number of clones and subjected to DNA sequence analysis. One clone having the correct DNA sequence was designated mHOB16 (Figure 4).

A molecule in which the mature HSA coding region was fused to the HSA secretion signal was created by insertion of Linker 4 into BamHI and XhoI digested M13mp19.7 to form pDBD2 (Figure 4).

Linker 4

```
                M     K     W     V     S     F
5' GATCC  ATG   AAG   TGG   GTA   AGC   TTT
      G   TAC   TTC   ACC   CAT   TCG   AAA

I     S     L     L     F     L     F     S
ATT   TCC   CTT   CTT   TTT   CTC   TTT   AGC
TAA   AGG   GAA   GAA   AAA   GAG   AAA   TCG
```

```
  S     A     Y     S     R     G     V     F
 TCG   GCT   TAT   TCC   AGG   GGT   GTG   TTT
 AGC   CGA   ATA   AGG   TCC   CCA   CAC   AAA

  R     R
 CG     3'
GCAGCT  5'
```

In this linker the codon for the fourth amino acid after the initial methionine, ACC for threonine in the HSA pre-pro leader sequence (Lawn et al, 1981), has been changed to AGC for serine to create a HindIII site.

A sequence of synthetic DNA representing a part of the known HSA coding sequence (Lawn et al., 1981) (amino acids 382 to 387, Fig. 2), fused to part of the known fibronectin coding sequence (Kornblihtt et al., 1985) (amino acids 585 to 640, Fig. 2), was prepared by synthesising six oligonucleotides (Linker 5, Fig. 6). The oligonucleotides 2, 3, 4, 6, 7 and 8 were phosphorylated using T4 polynucleotide kinasa and then tha oligonucleotides were annealed under standard conditions in pairs, i.e. 1+8, 2+7, 3+6 and 4+5. The annealed oligonucleotides wera then mixed together and ligated with mHOB12 which had previously been digested with the restriction enzymes HincII and EcoRI. The ligetion mixture was then used to transfect E.coli XL1-Blue (Stratagene Cloning Systems, San Diego, CA). Single stranded template DNA was then prepared from mature bacteriophage particles derived from several independent plaques and then was analysed by DNA sequencing. A clone in which a linker of tha expected sequence had been correctly inserted into the vector was designated pDBDF1 (Fig. 7). This plasmid was then digested with PstI and EcoRI and tha approx. 0.24kb fragment was purified and then ligated with the 1.29kb BamHI-PstI fragment of pDBD2 (Fig. 7) and BamHI + EcoRI digested pUC19 (Yanisch-Perron, et al., 1985) to form pDBDF2 (Fig. 7).

A plasmid containing a DNA sequence encoding full length human fibronectin, pFHDEL1, was digested with EcoRI and XhoI and a 0.77kb EcoRI-xhoI fragment (Fig. 8) was isolated and then ligated with EcoRI and sall digested M13 mp18 (Norrander et al., 1983) to form pDBDF3 (Fig. 8).

The following oligonucleotide linker (Linker 6) was synthesised, representing from the PstI site at 4784-4791 of the fibronectin sequence of EP-A-207 751 to the codon for tyrosine 1578 (Fig. 5) which is followed by a stop codon (TAA), a HindIII site and then a BamHI cohesive end:

Linker 6

```
          G   P   D   Q   T   E   M   T   I   E   G   L
        GGT CCA GAT CAA ACA GAA ATG ACT ATT GAA GGC TTG
  A CGT CCA GGT CTA GTT TGT CTT TAC TGA TAA CTT CCG AAC

  Q   P   T   V   E   Y       Stop
CAG CCC ACA GTG GAG TAT       TAA   GCTTG
GTC GGG TGT CAC CTC ATA       ATT   CGAACCTAG
```

This linker was then ligated with PstI and HindIII digested pDBDF3 to form pDBDF4 (Fig. 8). The following DNA fragments were then ligated together with BglII digested pKV50 (EP-A-258 067) as shown in Fig. 8: 0.68kb EcoRI-BamHI fragment of pDBDF4, 1.5kb BamHI-StuI fragment of pDBDF2 and the 2.2kb StuI-EcoRI fragment of pFHDEL1. The resultant plasmid pDBDF5 (Fig. 8) includes the promoter of EP-A-258 067 to direct the ex-

pression of the HSA secretion signal fusad to DNA encoding amino acids 1-387 of mature HSA, In turn fused directly and in frame with DNA encoding amino acids 585-1578 of human fibronectin, after which translation would terminate at the stop codon TAA. This is then followed by the S.cerevisiae PGK gena transcription terminator. The plasmid also contains sequences which permit selection and maintenance in Escherichia coli and S.cerevisiae (EP-A-258 067).

This plasmid was introduced into S.cerevisiae S150-2B (leu2-3 leu2-112 ura3-52 trp1-289 his3- 1) by standard procedures (Beggs, 1978). Transformants were subsequently analysed and found to produce the HSA-fibronectin fusion protein.

EXAMPLE 2 : HSA 1-195 FUSED TO Fn 585-1578

In this second example the first domain of human serum albumin (amino acids 1-195) is fusad to amino acids 585-1578 of human fibronectin.

The plasmid pDBD2 was digested with BamHI and BglII and the 0.79kb fragment was purified and then ligated with BamHI-digested M13mp19 to form pDBDF6 (Fig. 6). The following oligonucleotide:

5'-C C A A A G C T C G A G G A A C T T C G-3'

was used as e mutagenic primer to create a XhoI site in pDBDF6 by in vitro mutagenesis using a kit supplied by Amersham International PLC. This site was created by changing base number 696 of HSA from a T to a G (Fig. 2). The plasmid thus formed was designatad pDBDF7 (Fig. 9). The followlng llnker was then synthesised to represent from this newly creeted XhoI site to the codon for lysine 195 of HSA (AAA) and then from the codon for isoleucine 585 of fibronectin to the ends of oligonucleotides 1 and 8 shown In Fig. 6.

Linker 7

```
    D   E   L   R   D   E   G   K   A   S   S   A   K
TC GAT GAA CTT CGG GAT GAA GGG AAG GCT TCG TCT GCC AAA
     A CTT GAA GCC CTA CTT CCC TTC CGA AGC AGA CGG TTT

    I   T   E   T   P   S   Q   P   N   S   H
   ATC ACT GAG ACT CCG AGT CAG C
   TAG TGA CTC TGA GGC TCA GTC GGG TTG AGG GTG G
```

This linker was ligated with the annealed oligonucleotides shown in Fig. 3, i.e. 2+7, 3+6 and 4+5 together with XhoI and EcoRI digested pDBDF7 to form pDBDF8 (Fig. 9). Note that in order to recreate the original HSA DNA sequence, and hence amino acid sequence, insertion of linker 7 and tha other oligonucleotides into pDBDF7 does not recreate the XhoI site.

The 0.83kb BamHi-StuI fragment of pDBDF8 was purified and then was ligated with the 0.68kb EcoRI-BamHI fragment of pDBDF2 and the 2.22kb StuI-EcoRI fragment of pFHDEL1 into BglII-digested pKV50 to form pDBDF9 (Fig. 9). This plasmid is similar to pDBDF5 except that it specifies only residues 1-195 of HSA rather than 1-387 as in pDBDF5.

When introduced into S.cerevisiae S150-2B as above, the plasmid directed the expression and secretion of a hybrid molecule composed of residues 1-195 of HSA fused to residues 585-1578 of fibronectin.

EXAMPLE 3 : HSA 1-387 FUSED TO Fn 585-1578, AS CLEAVABLE MOLECULE

In order to facilitate production of large amounts of residues 585-1578 of fibronectin, a construct was made in which DNA encoding residues 1-387 of HSA was separated from DNA encoding residues 585-1578 of fibronectin by the sequence

```
I   E   G   R
ATT GAA GGT AGA
TAA CTT CCA TCT
```

which specifies the cleavage recognition site for the blood clotting Factor X. Consequently the purified secreted product can be treated with Factor X and then the fibronectin part of the molecule can be separated from the HSA part.

To do this two oligonucleotides were synthesised and then annealed to form Linker 8.

Linker 8

```
E   E   P   Q   N   L   I   E   G
GAA GAG CCT CAG AAT TTA ATT GAA GGT
CTT CTC GGA GTC TTA AAT TAA CTT CCA

R   I   T   E   T   P   S   Q   P
AGA ATC ACT GAG ACT CCG AGT CAG C
TCT TAG TGA CTC TGA GGC TCA GTC GGG

N   S   H

TTG AGG GTG G
```

This linker was then ligated with the annealed oligonucleotides shown in Fig. 6, i.e. 2+7, 3+6 and 4+5 into HincII and EcoRI digested mHOB12, to form pDBDF10 (Fig. 7). The plasmid was then digested with PstI and EcoRI and the roughly 0.24kb fragment was purified and then ligated with the 1.29kb BamHI-PstI fragment of pDBD2 and BamHI and EcoRI digested pUC19 to form pDBDF11 (Fig. 10).

The 1.5kb BamHI-StuI fragment of pDBDF11 was then ligated with the 0.68kb EcoRI-BamH1 fragment of pDBDF4 and the 2.22kb StuI-EcoRI fragment of pFHDEL1 into BglII-digested pKV50 to form pDBDF12 (Fig. 10). This plasmid was then introduced into S.cerevisiae S150-2B. The purified secreted fusion protein was treated with Factor X to liberate the fibronectin fragment representing residues 585-1578 of the native molecule.

REFERENCES

Beggs, J.D. (1978) Nature 275, 104-109

Kornblihtt et al. (1985) EMBO J. 4, 1755-1759

Lawn, R.M. et al. (1981) Nucl. Acid. Res. 9, 6103-6114

Maniatis, T. et al. (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Messing, J. (1983) Methods Enzymol. 101, 20-78

Norrander, J. et al. (1983) Gene 26, 101-106

Sanger, F. et al. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467

Yanisch-Perron, C. (1985) Gene 33, 103-119

Claims

Claims for the following Contracting States : AT, BE, CH, LI, DE, DK, FR, IT, LU, NL, SE

1. A fusion polypeptide comprising, as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof then the said polypeptide is (a) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, (c) platelet derived growth factor or a variant thereof, (d) transforming growth factor β or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alpha-1-antitrypsin or a variant thereof.

2. A fusion polypeptide according to Claim 1 additionally comprising at least one N-terminal amino acid extending beyond the portion corresponding to the N-terminal portion of HSA.

3. A fusion polypeptide according to Claim 1 or 2 wherein there is a cleaveble region at the junction of the said N-terminal or C-terminal portions.

4. A fusion polypeptide according to any one of the preceding claims wherein the said C-terminal portion is the 585 to 1578 portion of human plasma fibronectin or a variant thereof.

5. A transformed or transfected host having a nucleotide sequence so arranged as to express a fusion polypeptide according to any one of the preceding claims.

6. A process for preparing a fusion polypeptide by cultivation of a host according to Claim 5, followed by separation of the fusion polypeptide in a useful form.

7. A fusion polypeptide according to any one of Claims 1 to 4 for use in therapy.

Claims for the following Contracting States : ES, GR

1. A process for preparing a fusion polypeptide by (i) cultivation of a transformed or transfected host having a nucleotide sequence so arranged as to express a fusion polypeptide, followed by (ii) separation of the fusion polypeptide in a useful form, characterised in that the fusion polypeptide comprises as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof then the said polypeptide is (a) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, (c) platelet derived growth factor or a variant thereof, (d) transforming growth factor β or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alpha-1-antitrypsin or a variant thereof.

2. A process according to Claim 1, wherein the fusion polypeptide additionally comprising at least one N-terminal amino acid extending beyond the portion corresponding to the N-terminal portion of HSA.

3. A process according to Claim 1 or 2 wherein, in the fusion polypeptide, there is a cleavable region at the junction of the said N-terminal or C-terminal portions.

4. A process according to any one of the preceding claims wherein the said C-terminal portion is the 585 to 1578 portion of human plasma fibronectin or a variant thereof.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, DK, FR, IT, LU, NL, SE

1. Fusionspolypeptid, umfassend als mindestens einen Teil seines N-terminalen Teils einen N-terminalen Teil von HSA oder eine Variante devon und als mindestens einen Teil seines C-terminelen Teils ein weiteres Polypeptid mit der Ausnahme, daß wenn es sich bei dem N-terminelen Teil von HSA um den Teil 1-n mit n = 369 bis 419 oder eine Variante davon handelt, das Polypeptid aus
   (a) dem Teil 585 bis 1578 von Humanfibronectin oder einer Variante davon,
   (b) dem Tail 1 bis 368 von CD4 oder einer Variante davon,
   (c) dem "Platelet Derived Growth Factor" (PDGF) oder einer Variante davon,
   (d) dem "Transforming Growth Fector β" (TGF β) oder einer Variante davon,
   (e) dem Teil 1-261 von reifem Humanplasmafibronectin oder einer Veriante devon,
   (f) dem Teil 278-578 von reifem Humanplasmafibronectin oder einer Variante davon,
   (g) dem Teil 1-272 von reifem Human-von Willebrand's-Faktor oder einer Variante davon oder
   (h) Alpha-1-Antitrypsin oder einer Varianta davon, besteht.

2. Fusionspolypeptid nach Anspruch 1, zusätzlich umfassend mindestens eine N-terminale Aminosäure, die länger als der dem N-terminelen Teil von HSA entsprechende Teil ist.

3. Fusionspolypeptid nach Anspruch 1 oder 2, bei dem sich an der Verbindung der N-terminalen oder C-terminalen Teile eina spaltbare Region befindet.

4. Fusionspolypeptid nach einam der vorhergehenden Ansprüche, wobei dar C-terminale Teil aus dem Teil 585 bis 1578 von Humanplasmafibronectin oder einer Variante davon besteht.

5. Transformierter oder transfizierter Wirt mit einer Nukleotidsequenz, die so angeordnet ist, daß sie ein Fusionspolypeptid nach einem der vorhergehenden Ansprüche exprimieren kann.

6. Verfahren zur Herstellung eines Fusionspolypeptids durch Kultivieren eines Wirts nach Anspruch 5 und anschließendes Abtrennen des Fusionspolypeptids in einer geeigneten Form.

7. Fusionspolypeptid nach einem der Ansprücha 1 bis 4 zur therapeutischen Verwendung.

Patentansprüche für folgenda Vertragsstaatan : ES, GR

1. Verfahren zur Herstellung eines Fusionspolypeptids durch
   (i) Kultivieren eines transformierten oder transfektierten Wirts mit einer Nukleotidsequanz, die so angeordnet ist, daß sie ein Fusionspolypeptid exprimiert, und
   (ii) anschließendes Abtrennen des Fusionspolypeptids in einer geeigneten Form,
   dadurch gekennzeichnet, daß das Fusionspolypeptid als mindestens einen Teil seines N-terminalen Teils einen N-terminalen Teil von HSA oder eine Varianta davon und als mindestens ainen Teil seines C-terminalen Teils ein weiteres Polypeptid umfaßt, mit der Ausnahme, daß wenn es sich bei dem N-terminelen Teil von HSA um den Teil 1-n mit n= 369 bis 419 oder eine Variante davon handelt, das Polypeptid aus
   (a) dem Teil 585-1578 von Humanfibronectin oder einer Variante davon,
   (b) dem Teil 1-368 von CD4 oder einer Varianta davon,
   (c) dem Platelet Derived Growth Factor oder einer Variante davon,
   (d) dem Transforming Growth Factor β oder einer Variante davon,
   (e) dem Teil 1-261 von reifem Humanplasmafibronectin oder einer Variante davon,
   (f) dem Teil 278-578 von reifem Humanplasmafibronectin oder einer Variante devon,
   (g) dem Teil 1-272 von reifem Human-von Willebrand's-Faktor oder einer Variante davon oder
   (h) α-1-Antitrypsin oder einer Variante davon besteht.

2. Verfahren nach Anspruch 1, wobei das Fusionspolypeptid zusätzlich mindestens eine N-terminale Aminosäure, die länger als der dem N-terminalen Teil von HSA entsprechenda Teil ist, umfaßt.

3. Verfahren nach Anspruch 1 oder 2, wobei sich in dem Fusionspolypeptid an der Verbindung der N-terminalen oder C-terminalen Teile eine spaltbare Region befindet.

4. Verfahren nach einem der vorhergehenden Ansprüche, wobei der C-terminale Teil aus dem Teil 585-1578 von Humanplasmafibronectin oder einer Variante davon besteht.

Ravendications

Revendications pour las Etats contractants suivants : AT, BE, CH, DE, DK, FR, IT, LU, NL, SE

1. Polypeptida fusionné comprenant en tant qu'au moins une partie de sa portion N-terminale, une portion N-terminale de HSA ou d'un variant de celle-ci et, en tant qu'au molns une partie de sa portion C-tarminale, un autre polypeptide sauf que, lorsque cette portion N-terminale de HSA est la portion 1-n dans laquella n est 369 à 419 ou un variant de celle-ci, ce polypeptide est (a) la portion 585 à 1578 de la fibronectine humaine ou un variant de celle-ci, (b) la portion 1 à 368 de CD4 ou un variant de celle-ci, (c) le facteur de croissance dérivé des plaquettes sanguines ou un variant da celui-cl, (d) la facteur de croissance β de transformation ou un variant de celui-ci, (e) la portion 1-261 de la fibronectine meture de plasma humain ou un variant de celle-ci, (f) la portion 278-578 de la fibronectine matura de plasma humain ou un variant de celle-ci, (g) la portion 1-272 du facteur humaln mature de von Willebrand ou un variant de celle-ci, ou (h) l'alpha-1-antitrypsine ou un variant de celle-ci.

2. Polypeptide fusionné suivant la revendication 1, comprenant da plus au moins un acide aminé N-terminal se prolongeant au-delà de la portion correspondant à la portion N-terminale da HSA.

3. Polypeptide fusionné suivant les revendications 1 ou 2, dans lequel il y a une région susceptible d'être coupée à la jonction de ces portions N-terminale et C-terminale.

4. Polypeptide fusionné suivant l'une quelconque des revendications précédentes, dans lequel cette portion C-terminale est la portion 585 à 1578 de la fibronectine de plasma humain ou un variant de celle-ci.

5. Hôte transformé ou transfecté ayant une séquence de nucléotides arrangée de façon à exprimer un polypeptide fusionné suivant l'une quelconque des revendications précédentes.

6. Procédé pour préparer un polypeptide fuslonné par culture d'un hôte suivant la revendication 5, suivie de la séparation du polypeptide fusionné sous une forme utile.

7. Polypeptide fusionné suivant l'une quelconque des revendications 1 à 4 utilisable en thérapie.

Revendicatlons pour les Etats contractents suivants : ES, GR

1. Procédé pour préparer un polypeptide fusionné par (i) la culture d'un hôte transformé ou transfecté ayant une séquence de nucléotides arrangée de façon à exprimer un polypeptide fusionné, suivie de (ii) la séparation du polypeptide fusionné sous une forme utilie, caractérisé en ce que le polypeptide fusionné comprend, en tant qu'au moins une partie de se portion N-terminale, une portion N-terminale de HSA ou d'un varient de celle-ci et, en tant qu'au moins une partie de sa portion C-terminale, un autre polypeptide sauf que, lorsque cette portion N-terminale de HSA est la portion 1-n dans laquelle n est 369 à 419 ou un variant de celle-ci, ce polypeptide est alors (a) la portion 585 à 1578 de la fibronectine humaine ou un variant de celle-ci, (b) la portion 1 à 368 de CD4 ou un variant de celle-ci, (c) le facteur de croissance dérivé des plequettes sanguines ou un variant de celui-cl, (d) le facteur de croissanca β de transformation ou un variant de celui-ci, (e) la portion 1-261 de la fibronectine mature de plasma humain ou un variant de celle-ci, (f) la portion 278-578 de la fibronectine mature da plasma humain ou un variant de celle-ci, (g) la portion 1-272 du facteur humain mature de von Willebrand ou un variant de celle-ci, ou (h) l'alpha-1-antitrypsine ou un variant de celle-ci.

2. Procédé suivant la revendlcatlon 1, dans lequel le polypeptide fusionné comprend da plus au moins un acide aminé N-terminal se prolongeant au-delà de la portion correspondant é la portion N-terminale de HSA.

3. Procédé suivant les revendications 1 ou 2 dans lequel, dans le polypeptide fusionné, il y a une région susceptible d'être coupée à la jonction de ces portions N-terminale et C-terminale.

4. Procédé suivant l'une quelconque des revendications précédentes, dens lequel cette portion C-terminele est la portion 585 à 1578 de la fibronectine de plasma humain ou un variant de celle-ci.

FIGURE 1

```
                                                                    10                                                                  20
Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu Glu Asn Phe Lys

                                                                    30                                                                  40
Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val

                                                                    50                                                                  60
Lys Leu Val Asn Glu Val Thr Glu Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu

                                                                    70                                                                  80
Asn Cys Asp Lys Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu

                                                                    90                                                                  100
Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro Glu Arg Asn Glu

                                                                    110                                                                 120
Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu Pro Arg Leu Val Arg Pro Glu Val

                                                                    130                                                                 140
Asp Val Met Cys Thr Ala Phe His Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr

                                                                    150                                                                 160
Glu Ile Ala Arg Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg

                                                                    170                                                                 180
Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala Cys Leu Leu Pro

                                                                    190                                                                 200
Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser Ser Ala Lys Gln Arg Leu Lys Cys

                                                                    210                                                                 220
Ala Ser Leu Gln Lys Phe Gly Glu Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser

                                                                    230                                                                 240
Gln Arg Phe Pro Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys

                                                                    250                                                                 260
Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp Arg Ala Asp Leu

                                                                    270                                                                 280
Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser Ser Lys Leu Lys Glu Cys Cys Glu

                                                                    290                                                                 300
Lys Pro Leu Leu Glu Lys Ser His Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala

                                                                    310                                                                 320
Asp Leu Pro Ser Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala

                                                                    330                                                                 340
Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg Arg His Pro Asp

                                                                    350                                                                 360
Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr Tyr Glu Thr Thr Leu Glu Lys Cys

                                                                    370                                                                 380
Cys Ala Ala Ala Asp Pro His Glu Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu
```

FIGURE 1 Cont.

390 400
Val Glu Glu Pro Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu

410 420
Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro Gln Val Ser Thr

430 440
Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys Val Gly Ser Lys Cys Cys Lys His

450 460
Pro Glu Ala Lys Arg Met Pro Cys Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu

470 480
Cys Val Leu His Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser

490 500
Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr Tyr Val Pro Lys

510 520
Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp Ile Cys Thr Leu Ser Glu Lys Glu

530 540
Arg Gln Ile Lys Lys Gln Thr Ala Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr

550 560
Lys Glu Gln Leu Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys

570 580
Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val Ala Ala Ser Gln

Ala Ala Leu Gly Leu

FIGURE 2 DNA sequence coding for mature HSA

```
        10         20         30         40         50         60         70         80
GATGCACACAAGAGTGAGGTTGCTCATCGGTTTAAAGATTTGGGAGAAGAAAATTTCAAAGCCTTGGTGTTGATTGCCTT
 D  A  H  K  S  E  V  A  H  R  F  K  D  L  G  E  E  N  F  K  A  L  V  L  I  A  F

        90        100        110        120        130        140        150        160
TGCTCAGTATCTTCAGCAGTGTCCATTTGAAGATCATGTAAAATTAGTGAATGAAGTAACTGAATTTGCAAAAACATGTG
  A  Q  Y  L  Q  Q  C  P  F  E  D  H  V  K  L  V  N  E  V  T  E  F  A  K  T  C

       170        180        190        200        210        220        230        240
TTGCTGATGAGTCAGCTGAAAATTGTGACAAATCACTTCATACCCTTTTTGGAGACAAATTATGCACAGTTGCAACTCTT
 V  A  D  E  S  A  E  N  C  D  K  S  L  H  T  L  F  G  D  K  L  C  T  V  A  T  L

       250        260        270        280        290        300        310        320
CGTGAAACCTATGGTGAAATGGCTGACTGCTGTGCAAAACAAGAACCTGAGAGAAATGAATGCTTCTTGCAACACAAAGA
 R  E  T  Y  G  E  M  A  D  C  C  A  K  Q  E  P  E  R  N  E  C  F  L  Q  H  K  D

       330        340        350        360        370        380        390        400
TGACAACCCAAACCTCCCCCGATTGGTGAGACCAGAGGTTGATGTGATGTGCACTGCTTTTCATGACAATGAAGAGACAT
  D  N  P  N  L  P  R  L  V  R  P  E  V  D  V  M  C  T  A  F  H  D  N  E  E  T

       410        420        430        440        450        460        470        480
TTTTGAAAAAATACTTATATGAAATTGCCAGAAGACATCCTTACTTTTATGCCCCCGGAACTCCTTTTCTTTGCTAAAAGG
 F  L  K  K  Y  L  Y  E  I  A  R  R  H  P  Y  F  Y  A  P  E  L  L  F  F  A  K  R

       490        500        510        520        530        540        550        560
TATAAAGCTGCTTTTACAGAATGTGCCAAGCTGCTGATAAAGCTGCCTGCCTGTTGCCAAAGCTCGATGAACTTCGGGA
 Y  K  A  A  F  T  E  C  C  Q  A  A  D  K  A  A  C  L  L  P  K  L  D  E  L  R  D

       570        580        590        600        610        620        630        640
TGAAGGGAAGGCTTCGTCTGCCAAACAGAGACTCAAATGTGCCAGTCTCCAAAAATTTGGAGAAAGAGCTTTCAAAGCAT
  E  G  K  A  S  S  A  K  Q  R  L  K  C  A  S  L  Q  K  F  G  E  R  A  F  K  A

       650        660        670        680        690        700        710        720
GGGCAGTGGCTCGCCTGAGCCAGAGATTTCCCAAAGCTGAGTTTGCAGAAGTTTCCAAGTTAGTGACAGATCTTACCAAA
 W  A  V  A  R  L  S  Q  R  F  P  K  A  E  F  A  E  V  S  K  L  V  T  D  L  T  K

       730        740        750        760        770        780        790        800
GTCCACACGGAATGCTGCCATGGAGATCTGCTTGAATGTGCTGATGACAGGGCGGACCTTGCCAAGTATATCTGTGAAAA
 V  H  T  E  C  C  H  G  D  L  L  E  C  A  D  D  R  A  D  L  A  K  Y  I  C  E  N

       810        820        830        840        850        860        870        880
TCAGGATTCGATCTCCAGTAAACTGAAGGAATGCTGTGAAAAACCTCTGTTGGAAAAATCCCACTGCATTGCCGAAGTGG
  Q  D  S  I  S  S  K  L  K  E  C  C  E  K  P  L  L  E  K  S  H  C  I  A  E  V

       890        900        910        920        930        940        950        960
AAAATGATGAGATGCCTGCTGACTTGCCTTCATTAGCTGCTGATTTTGTTGAAAGTAAGGATGTTTGCAAAAACTATGCT
 E  N  D  E  M  P  A  D  L  P  S  L  A  A  D  F  V  E  S  K  D  V  C  K  N  Y  A

       970        980        990       1000       1010       1020       1030       1040
GAGGCAAAGGATGTCTTCCTGGGCATGTTTTTGTATGAATATGCAAGAAGGCATCCTGATTACTCTGTCGTGCTGCTGCT
 E  A  K  D  V  F  L  G  M  F  L  Y  E  Y  A  R  R  H  P  D  Y  S  V  V  L  L  L
```

FIGURE 2 Cont.

```
      1050       1060       1070       1080       1090       1100       1110       1120
GAGACTTGCCAAGACATATGAAACCACTCTAGAGAAGTGCTGTGCCGCTGCAGATCCTCATGAATGCTATGCCAAAGTGT
  R  L  A  K  T  Y  E  T  T  L  E  K  C  C  A  A  A  D  P  H  E  C  Y  A  K  V

      1130       1140       1150       1160       1170       1180       1190       1200
TCGATGAACTTTAAACCTCTTGTGGAAGAGCCTCAGAATTTAATCAAACAAAACTGTGAGCTTTTTGAGCAGCTTGGAGAG
F  D  E  F  K  P  L  V  E  E  P  Q  N  L  I  K  Q  N  C  E  L  F  E  Q  L  G  E

      1210       1220       1230       1240       1250       1260       1270       1280
TACAAATTCCAGAATGCGCTATTAGTTCGTTACACCAAGAAAGTACCCCAAGTGTCAACTCCAACTCTTGTAGAGGTCTC
 Y  K  F  Q  N  A  L  L  V  R  Y  T  K  K  V  P  Q  V  S  T  P  T  L  V  E  V  S

      1290       1300       1310       1320       1330       1340       1350       1360
AAGAAACCTAGGAAAAGTGGGCAGCAAATGTTGTAAACATCCTGAAGCAAAAAGAATGCCCTGTGCAGAAGACTATCTAT
  R  N  L  G  K  V  G  S  K  C  C  K  H  P  E  A  K  R  M  P  C  A  E  D  Y  L

      1370       1380       1390       1400       1410       1420       1430       1440
CCGTGGTCCTGAACCAGTTATGTGTGTTGCATGAGAAAACGCCAGTAAGTGACAGAGTCACAAAATGCTGCACAGAGTCC
S  V  V  L  N  Q  L  C  V  L  H  E  K  T  P  V  S  D  R  V  T  K  C  C  T  E  S

      1450       1460       1470       1480       1490       1500       1510       1520
TTGGTGAACAGGCGACCATGCTTTTCAGCTCTGGAAGTCGATGAAACATACGTTCCCAAAGAGTTTAATGCTGAAACATT
 L  V  N  R  R  P  C  F  S  A  L  E  V  D  E  T  Y  V  P  K  E  F  N  A  E  T  F

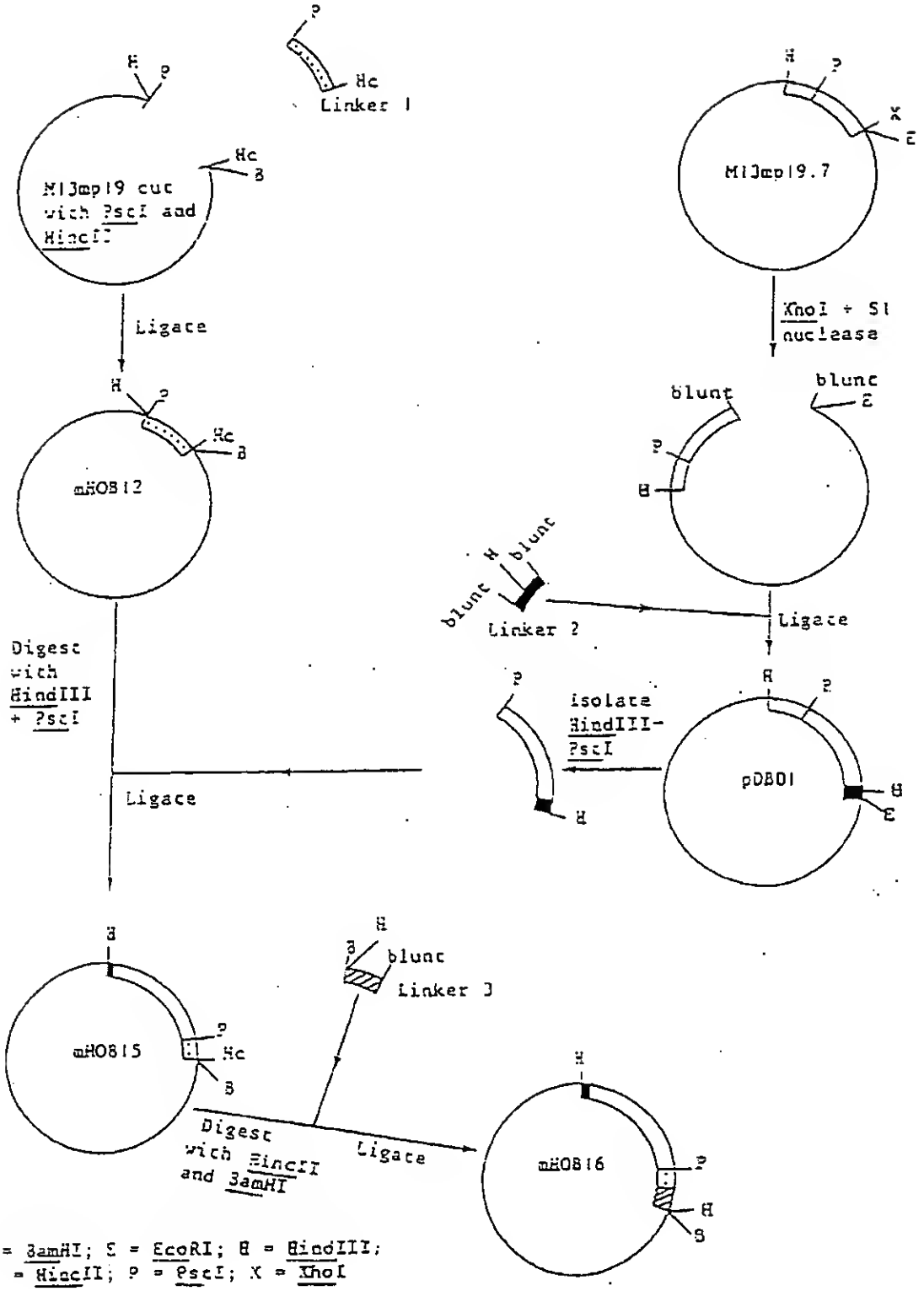
      1530       1540       1550       1560       1570       1580       1590       1600
CACCTTCCATGCAGATATATGCACACTTTCTGAGAAGGAGAGACAAATCAAGAAACAAACTGCACTTGTTGAGCTTGTGA
  T  F  H  A  D  I  C  T  L  S  E  K  E  R  Q  I  K  K  Q  T  A  L  V  E  L  V

      1610       1620       1630       1640       1650       1660       1670       1680
AACACAAGCCCAAGGCAACAAAAGAGCAACTGAAAGCTGTTATGGATGATTTCGCAGCTTTTGTAGAGAAGTGCTGCAAG
K  H  K  P  K  A  T  K  E  Q  L  K  A  V  M  D  D  F  A  A  F  V  E  K  C  C  K

      1690       1700       1710       1720       1730       1740       1750       1760
GCTGACGATAAGGAGACCTGCTTTGCCGAGGAGGGTAAAAAACTTGTTGCTGCAAGTCAAGCTGCCTTAGGCTTATAACA
 A  D  D  K  E  T  C  F  A  E  E  G  K  K  L  V  A  A  S  Q  A  A  L  G  L

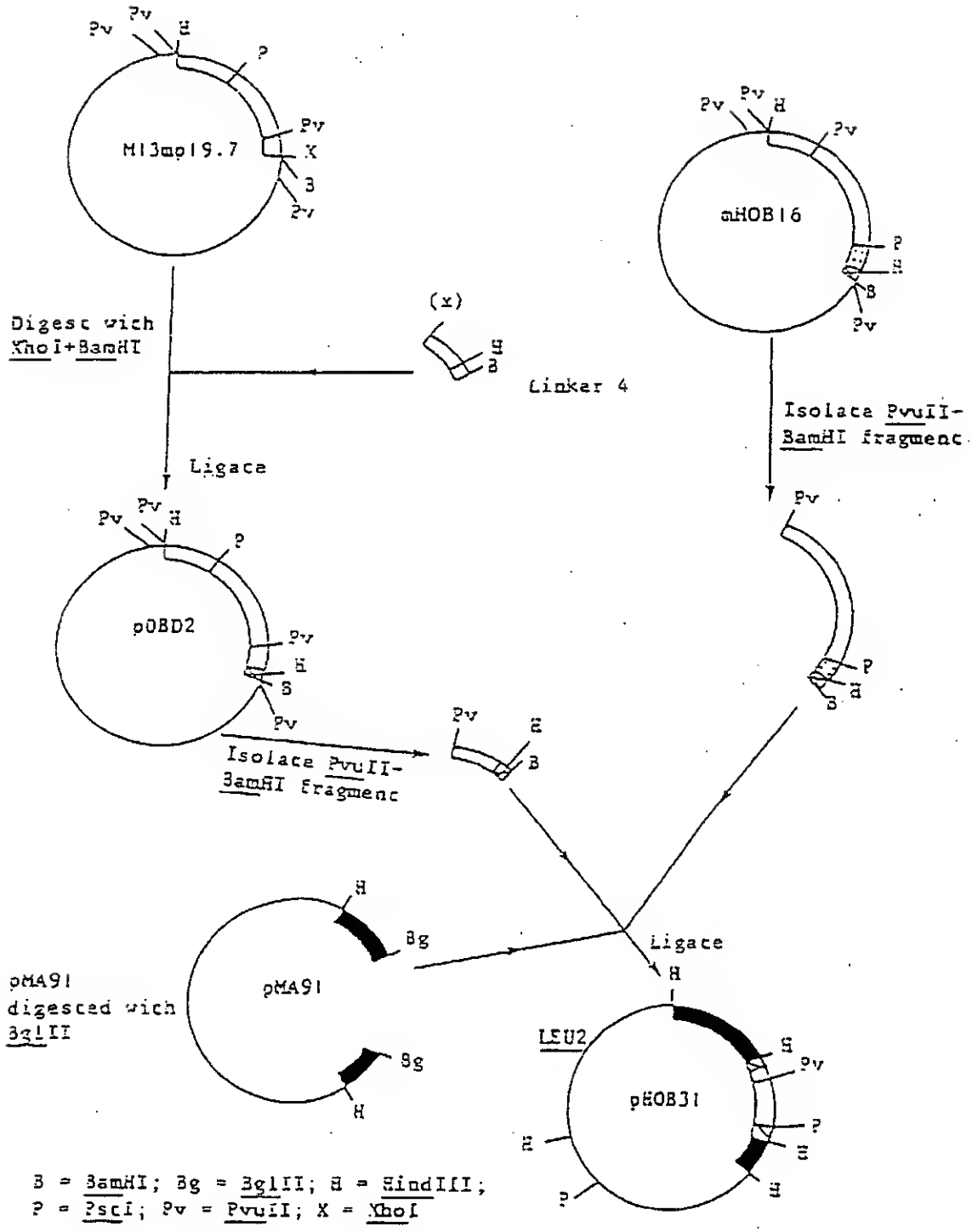
      1770       1780
TCTACATTTAAAAGCATCTCAG
```

FIGURE 3 Construction of mHO816



B = BamHI; S = EcoRI; H = HindIII;
Hc = HincII; P = PstI; X = XhoI

FIGURE 4 Construction of pHOB31



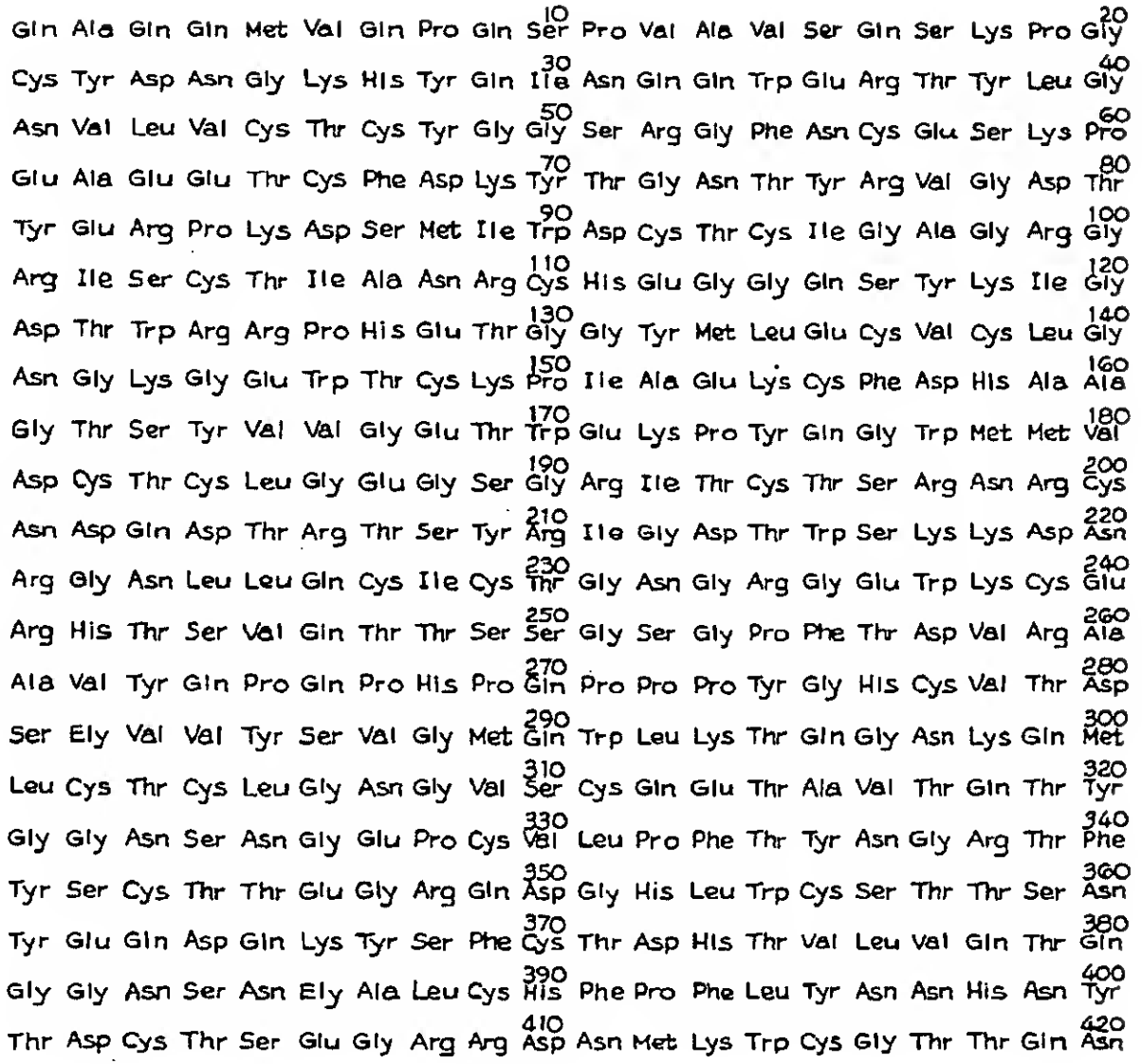

Gln Ala Gln Gln Met Val Gln Pro Gln Ser Pro Val Ala Val Ser Gln Ser Lys Pro Gly
Cys Tyr Asp Asn Gly Lys His Tyr Gln Ile Asn Gln Gln Trp Glu Arg Thr Tyr Leu Gly
Asn Val Leu Val Cys Thr Cys Tyr Gly Gly Ser Arg Gly Phe Asn Cys Glu Ser Lys Pro
Glu Ala Glu Glu Thr Cys Phe Asp Lys Tyr Thr Gly Asn Thr Tyr Arg Val Gly Asp Thr
Tyr Glu Arg Pro Lys Asp Ser Met Ile Trp Asp Cys Thr Cys Ile Gly Ala Gly Arg Gly
Arg Ile Ser Cys Thr Ile Ala Asn Arg Cys His Glu Gly Gly Gln Ser Tyr Lys Ile Gly
Asp Thr Trp Arg Arg Pro His Glu Thr Gly Gly Tyr Met Leu Glu Cys Val Cys Leu Gly
Asn Gly Lys Gly Glu Trp Thr Cys Lys Pro Ile Ala Glu Lys Cys Phe Asp His Ala Ala
Gly Thr Ser Tyr Val Val Gly Glu Thr Trp Glu Lys Pro Tyr Gln Gly Trp Met Met Val
Asp Cys Thr Cys Leu Gly Glu Gly Ser Gly Arg Ile Thr Cys Thr Ser Arg Asn Arg Cys
Asn Asp Gln Asp Thr Arg Thr Ser Tyr Arg Ile Gly Asp Thr Trp Ser Lys Lys Asp Asn
Arg Gly Asn Leu Leu Gln Cys Ile Cys Thr Gly Asn Gly Arg Gly Glu Trp Lys Cys Glu
Arg His Thr Ser Val Gln Thr Thr Ser Ser Gly Ser Gly Pro Phe Thr Asp Val Arg Ala
Ala Val Tyr Gln Pro Gln Pro His Pro Gln Pro Pro Pro Tyr Gly His Cys Val Thr Asp
Ser Gly Val Val Tyr Ser Val Gly Met Gln Trp Leu Lys Thr Gln Gly Asn Lys Gln Met
Leu Cys Thr Cys Leu Gly Asn Gly Val Ser Cys Gln Glu Thr Ala Val Thr Gln Thr Tyr
Gly Gly Asn Ser Asn Gly Glu Pro Cys Val Leu Pro Phe Thr Tyr Asn Gly Arg Thr Phe
Tyr Ser Cys Thr Thr Glu Gly Arg Gln Asp Gly His Leu Trp Cys Ser Thr Thr Ser Asn
Tyr Glu Gln Asp Gln Lys Tyr Ser Phe Cys Thr Asp His Thr Val Leu Val Gln Thr Gln
Gly Gly Asn Ser Asn Gly Ala Leu Cys His Phe Pro Phe Leu Tyr Asn Asn His Asn Tyr
Thr Asp Cys Thr Ser Glu Gly Arg Arg Asp Asn Met Lys Trp Cys Gly Thr Thr Gln Asn

Fig. 5A

Tyr Asp Ala Asp Gln Lys Phe Gly Phe 430 Cys Pro Met Ala Ala His Glu Glu Ile Cys 440 Thr
Thr Asn Glu Gly Val Met Tyr Arg Ile 450 Gly Asp Gln Trp Asp Lys Gln His Asp Met 460 Gly
His Met Met Arg Cys Thr Cys Val Gly 470 Asn Gly Arg Gly Glu Trp Thr Cys Tyr Ala 480 Tyr
Ser Gln Leu Arg Asp Gln Cys Ile Val 490 Asp Asp Ile Thr Tyr Asn Val Asn Asp Thr 500 Phe
His Lys Arg His Glu Glu Gly His Met 510 Leu Asn Cys Thr Cys Phe Gly Gln Gly Arg 520 Gly
Arg Trp Lys Cys Asp Pro Val Asp Gln 530 Cys Gln Asp Ser Glu Thr Gly Thr Phe Tyr 540 Gln
Ile Gly Asp Ser Trp Glu Lys Tyr Val 550 His Gly Val Arg Tyr Gln Cys Tyr Cys Tyr 560 Gly
Arg Gly Ile Gly Glu Trp His Cys Gln 570 Pro Leu Gln Thr Tyr Pro Ser Ser Ser Gly 580 Pro
Val Glu Val Phe Ile Thr Glu Thr Pro 590 Ser Gln Pro Asn Ser His Pro Ile Gln Trp 600 Asn
Ala Pro Gln Pro Ser His Ile Ser Lys 610 Tyr Ile Leu Arg Trp Arg Pro Lys Asn Ser 620 Val
Gly Arg Trp Lys Glu Ala Thr Ile Pro 630 Gly His Leu Asn Ser Tyr Thr Ile Lys Gly 640 Leu
Lys Pro Gly Val Val Tyr Glu Gly Gln 650 Leu Ile Ser Ile Gln Gln Tyr Gly His Gln 660 Glu
Val Thr Arg Phe Asp Phe Thr Thr Thr 670 Ser Thr Ser Thr Pro Val Thr Ser Asn Thr 680 Val
Thr Gly Glu Thr Thr Pro Phe Ser Pro 690 Leu Val Ala Thr Ser Glu Ser Val Thr Glu 700 Ile
Thr Ala Ser Ser Phe Val Val Ser Trp 710 Val Ser Ala Ser Asp Thr Val Ser Gly Phe 720 Arg
Val Glu Tyr Glu Leu Ser Glu Glu Gly 730 Asp Glu Pro Gln Tyr Leu Asp Leu Pro Ser 740 Thr
Ala Thr Ser Val Asn Ile Pro Asp Leu 750 Leu Pro Gly Arg Lys Tyr Ile Val Asn Val 760 Tyr
Gln Ile Ser Glu Asp Gly Glu Gln Ser 770 Leu Ile Leu Ser Thr Ser Gln Thr Thr Ala 780 Pro
Asp Ala Pro Pro Asp Pro Thr Val Asp 790 Gln Val Asp Asp Thr Ser Ile Val Val Arg 800 Trp
Ser Arg Pro Gln Ala Pro Ile Thr Gly 810 Tyr Arg Ile Val Tyr Ser Pro Ser Val Glu 820 Gly
Ser Ser Thr Glu Leu Asn Leu Pro Glu 830 Thr Ala Asn Ser Val Thr Leu Ser Asp Leu 840 Gln

FNDEL 1

Fig. 5B

Pro Gly Val Gln Tyr Asn Ile Thr Ile 850 Tyr Ala Val Glu Glu Asn Gln Glu Ser Thr 860 Pro
Val Val Ile Gln Gln Glu Thr Thr Gly 870 Thr Pro Arg Ser Asp Thr Val Pro Ser Pro 880 Arg
Asp Leu Gln Phe Val Glu Val Thr Asp 890 Val Lys Val Thr Ile Met Trp Thr Pro Pro 900 Glu
Ser Ala Val Thr Gly Tyr Arg Val Asp 910 Val Ile Pro Val Asn Leu Pro Gly Glu His 920 Gly
Gln Arg Leu Pro Ile Ser Arg Asn Thr 930 Phe Ala Glu Val Thr Gly Leu Ser Pro Gly 940 Val
Thr Tyr Tyr Phe Lys Val Phe Ala Val 950 Ser His Gly Arg Glu Ser Lys Pro Leu Thr 960 Ala
Gln Gln Thr Thr Lys Leu Asp Ala Pro 970 Thr Asn Leu Gln Phe Val Asn Glu Thr Asp 980 Ser
Thr Val Leu Val Arg Trp Thr Pro Pro 990 Arg Ala Gln Ile Thr Gly Tyr Arg Leu Thr 1000 Val
Gly Leu Thr Arg Arg Gly Gln Pro Arg 1010 Gln Tyr Asn Val Gly Pro Ser Val Ser Lys 1020 Tyr
Pro Leu Arg Asn Leu Gln Pro Ala Ser 1030 Glu Tyr Thr Val Ser Leu Val Ala Ile Lys 1040 Gly
Asn Gln Glu Ser Pro Lys Ala Thr Gly 1050 Val Phe Thr Thr Leu Gln Pro Gly Ser Ser 1060 Ile
Pro Pro Tyr Asn Thr Glu Val Thr Glu 1070 Thr Thr Ile Val Ile Thr Trp Thr Pro Ala 1080 Pro
Arg Ile Gly Phe Lys Leu Gly Val Arg 1090 Pro Ser Gln Gly Gly Glu Ala Pro Arg Glu 1100 Val
Thr Ser Asp Ser Gly Ser Ile Val Val 1110 Ser Gly Leu Thr Pro Gly Val Glu Tyr Val 1120 Tyr
Thr Ile Gln Val Leu Arg Asp Gly Gln 1130 Glu Arg Asp Ala Pro Ile Val Asn Lys Val 1140 Val
Thr Pro Leu Ser Pro Pro Thr Asn Leu 1150 His Leu Glu Ala Asn Pro Asp Thr Gly Val 1160 Leu
Thr Val Ser Trp Glu Arg Ser Thr Thr 1170 Pro Asp Ile Thr Gly Tyr Arg Ile Thr Thr 1180 Thr
Pro Thr Asn Gly Gln Gln Gly Asn Ser 1190 Leu Glu Glu Val Val His Ala Asp Gln Ser 1200 Ser
Cys Thr Phe Asp Asn Leu Ser Pro Gly 1210 Leu Glu Tyr Asn Val Ser Val Tyr Thr Val 1220 Lys
Asp Asp Lys Glu Ser Val Pro Ile Ser 1230 Asp Thr Ile Ile Pro Ala Val Pro Pro Pro 1240 Thr
Asp Leu Arg Phe Thr Asn Ile Gly Pro 1250 Asp Thr Met Arg Val Thr Trp Ala Pro Pro 1260 Pro

Fig. 5C

Ser Ile Asp Leu Thr Asn Phe Leu Val 1270 Arg Tyr Ser Pro Val Lys Asn Glu Glu Asp 1280 Val
Ala Glu Leu Ser Ile Ser Pro Ser Asp 1290 Asn Ala Val Val Leu Thr Asn Leu Leu Pro 1300 Gly
Thr Glu Tyr Val Val Ser Val Ser Ser 1310 Val Tyr Glu Gln His Glu Ser Thr Pro Leu 1320 Arg
Gly Arg Gln Lys Thr Gly Leu Asp Ser 1330 Pro Thr Gly Ile Asp Phe Ser Asp Ile Thr 1340 Ala
Asn Ser Phe Thr Val His Trp Ile Ala 1350 Pro Arg Ala Thr Ile Thr Gly Tyr Arg Ile 1360 Arg
His His Pro Glu His Phe Ser Gly Arg 1370 Pro Arg Glu Asp Arg Val Pro His Ser Arg 1380 Asn
Ser Ile Thr Leu Thr Asn Leu Thr Pro 1390 Gly Thr Glu Tyr Val Val Ser Ile Val Ala 1400 Leu
Asn Gly Arg Glu Glu Ser Pro Leu Leu 1410 Ile Gly Gln Gln Ser Thr Val Ser Asp Val 1420 Pro
Arg Asp Leu Glu Val Val Ala Ala Thr 1430 Pro Thr Ser Leu Leu Ile Ser Trp Asp Ala 1440 Pro
Ala Val Thr Val Arg Tyr Tyr Arg Ile 1450 Thr Tyr Gly Glu Thr Gly Gly Asn Ser Pro 1460 Val
Gln Glu Phe Thr Val Pro Gly Ser Lys 1470 Ser Thr Ala Thr Ile Ser Gly Leu Lys Pro 1480 Gly
Val Asp Tyr Thr Ile Thr Val Tyr Ala 1490 Val Thr Gly Arg Gly Asp Ser Pro Ala Ser 1500 Ser
Lys Pro Ile Ser Ile Asn Tyr Arg Thr 1510 Glu Ile Asp Lys Pro Ser Gln Met Gln Val 1520 Thr
Asp Val Gln Asp Asn Ser Ile Ser Val 1530 Lys Trp Leu Pro Ser Ser Ser Pro Val Thr 1540 Gly
Tyr Arg Val Thr Thr Thr Pro Lys Asn 1550 Gly Pro Gly Pro Thr Lys Thr Lys Thr Ala 1560 Gly
Pro Asp Gln Thr Glu Met Thr Ile Glu 1570 Gly Leu Gln Pro Thr Val Glu Tyr Val Val 1580 Ser
Val Tyr Ala Gln Asn Pro Ser Gly Glu 1590 Ser Gln Pro Leu Val Gln Thr Ala Val Thr 1600 Thr
Ile Pro Ala Pro Thr Asp Leu Lys Phe 1610 Thr Gln Val Thr Pro Thr Ser Leu Ser Ala 1620 Gln
Trp Thr Pro Pro Asn Val Gln Leu Thr 1630 Gly Tyr Arg Val Arg Val Thr Pro Lys Glu 1640 Lys
Thr Gly Pro Met Lys Glu Ile Asn Leu 1650 Ala Pro Asp Ser Ser Ser Val Val Val Ser 1660 Gly
Leu Met Val Ala Thr Lys Tyr Glu Val 1670 Ser Val Tyr Ala Leu Lys Asp Thr Leu Thr 1680 Ser
FNDEL 1

Fig. 5D

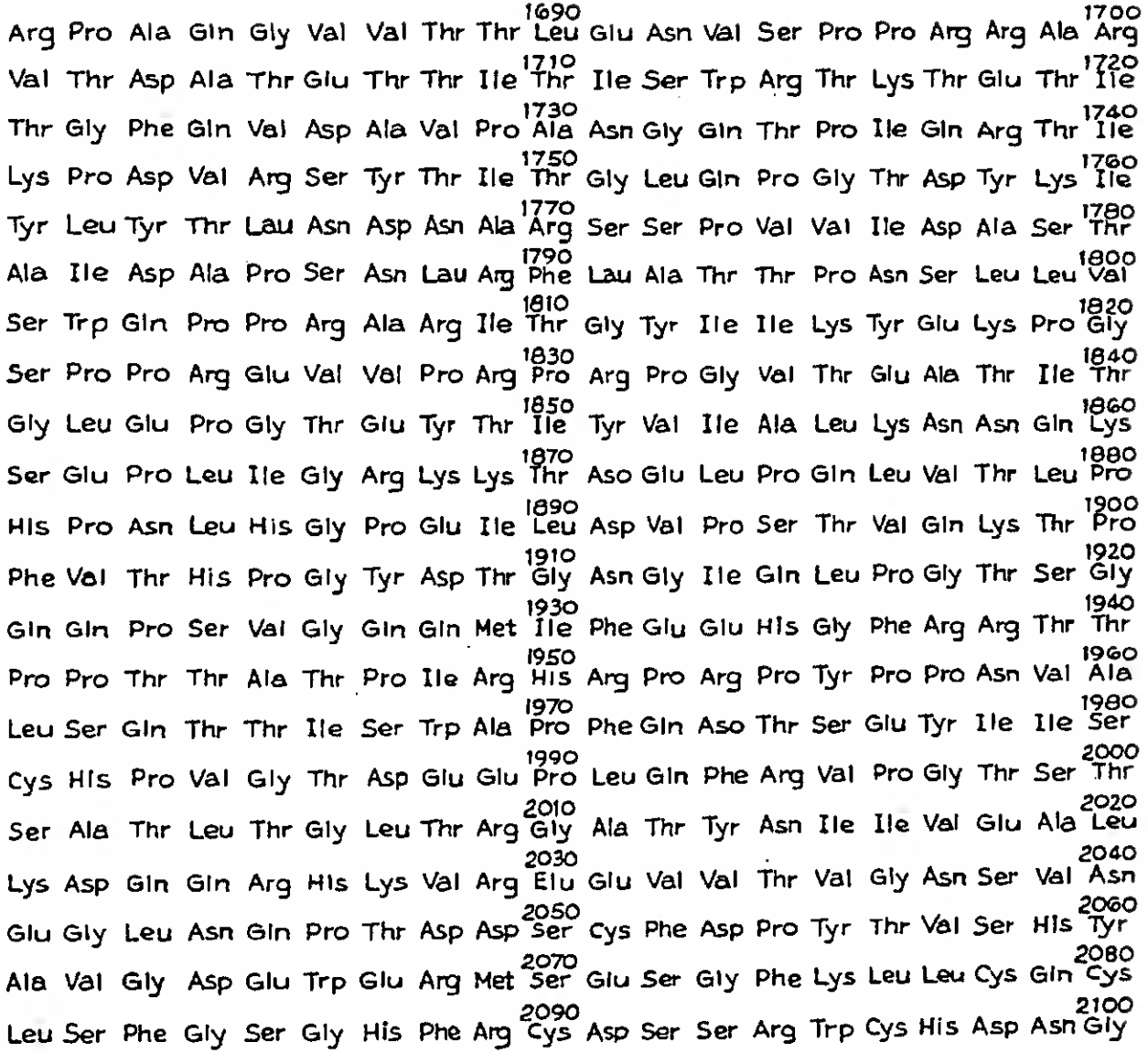

1690 1700
Arg Pro Ala Gln Gly Val Val Thr Thr Leu Glu Asn Val Ser Pro Pro Arg Arg Ala Arg
1710 1720
Val Thr Asp Ala Thr Glu Thr Thr Ile Thr Ile Ser Trp Arg Thr Lys Thr Glu Thr Ile
1730 1740
Thr Gly Phe Gln Val Asp Ala Val Pro Ala Asn Gly Gln Thr Pro Ile Gln Arg Thr Ile
1750 1760
Lys Pro Asp Val Arg Ser Tyr Thr Ile Thr Gly Leu Gln Pro Gly Thr Asp Tyr Lys Ile
1770 1780
Tyr Leu Tyr Thr Leu Asn Asp Asn Ala Arg Ser Ser Pro Val Val Ile Asp Ala Ser Thr
1790 1800
Ala Ile Asp Ala Pro Ser Asn Leu Arg Phe Leu Ala Thr Thr Pro Asn Ser Leu Leu Val
1810 1820
Ser Trp Gln Pro Pro Arg Ala Arg Ile Thr Gly Tyr Ile Ile Lys Tyr Glu Lys Pro Gly
1830 1840
Ser Pro Pro Arg Glu Val Val Pro Arg Pro Arg Pro Gly Val Thr Glu Ala Thr Ile Thr
1850 1860
Gly Leu Glu Pro Gly Thr Glu Tyr Thr Ile Tyr Val Ile Ala Leu Lys Asn Asn Gln Lys
1870 1880
Ser Glu Pro Leu Ile Gly Arg Lys Lys Thr Asp Glu Leu Pro Gln Leu Val Thr Leu Pro
1890 1900
His Pro Asn Leu His Gly Pro Glu Ile Leu Asp Val Pro Ser Thr Val Gln Lys Thr Pro
1910 1920
Phe Val Thr His Pro Gly Tyr Asp Thr Gly Asn Gly Ile Gln Leu Pro Gly Thr Ser Gly
1930 1940
Gln Gln Pro Ser Val Gly Gln Gln Met Ile Phe Glu Glu His Gly Phe Arg Arg Thr Thr
1950 1960
Pro Pro Thr Thr Ala Thr Pro Ile Arg His Arg Pro Arg Pro Tyr Pro Pro Asn Val Ala
1970 1980
Leu Ser Gln Thr Thr Ile Ser Trp Ala Pro Phe Gln Asp Thr Ser Glu Tyr Ile Ile Ser
1990 2000
Cys His Pro Val Gly Thr Asp Glu Glu Pro Leu Gln Phe Arg Val Pro Gly Thr Ser Thr
2010 2020
Ser Ala Thr Leu Thr Gly Leu Thr Arg Gly Ala Thr Tyr Asn Ile Ile Val Glu Ala Leu
2030 2040
Lys Asp Gln Gln Arg His Lys Val Arg Glu Glu Val Val Thr Val Gly Asn Ser Val Asn
2050 2060
Glu Gly Leu Asn Gln Pro Thr Asp Asp Ser Cys Phe Asp Pro Tyr Thr Val Ser His Tyr
2070 2080
Ala Val Gly Asp Glu Trp Glu Arg Met Ser Glu Ser Gly Phe Lys Leu Leu Cys Gln Cys
2090 2100
Leu Ser Phe Gly Ser Gly His Phe Arg Cys Asp Ser Ser Arg Trp Cys His Asp Asn Gly

Fig. 5E

Val Asn Tyr Lys Ile Gly Glu Lys Trp Asp Arg Gln Gly Glu Asn Gly Gln Met Met Ser
2110 2120
Cys Thr Cys Leu Gly Asn Gly Lys Gly Glu Phe Lys Cys Asp Pro His Glu Ala Thr Cys
2130 2140
Tyr Asp Asp Gly Lys Thr Tyr His Val Gly Glu Gln Trp Gln Lys Glu Tyr Leu Gly Ala
2150 2160
Ile Cys Ser Cys Thr Cys Phe Gly Gly Gln Arg Gly Trp Arg Cys Asp Asn Cys Arg Arg
2170 2180
Pro Gly Gly Glu Pro Ser Pro Glu Gly Thr Thr Gly Gln Ser Tyr Asn Gln Tyr Ser Gln
2190 2200
Arg Tyr His Gln Arg Thr Asn Thr Asn Val Asn Cys Pro Ile Glu Cys Phe Met Pro Leu
2210 2220
Asp Val Gln Ala Asp Arg Glu Asp Ser Arg Glu
2230

Fig. 5F

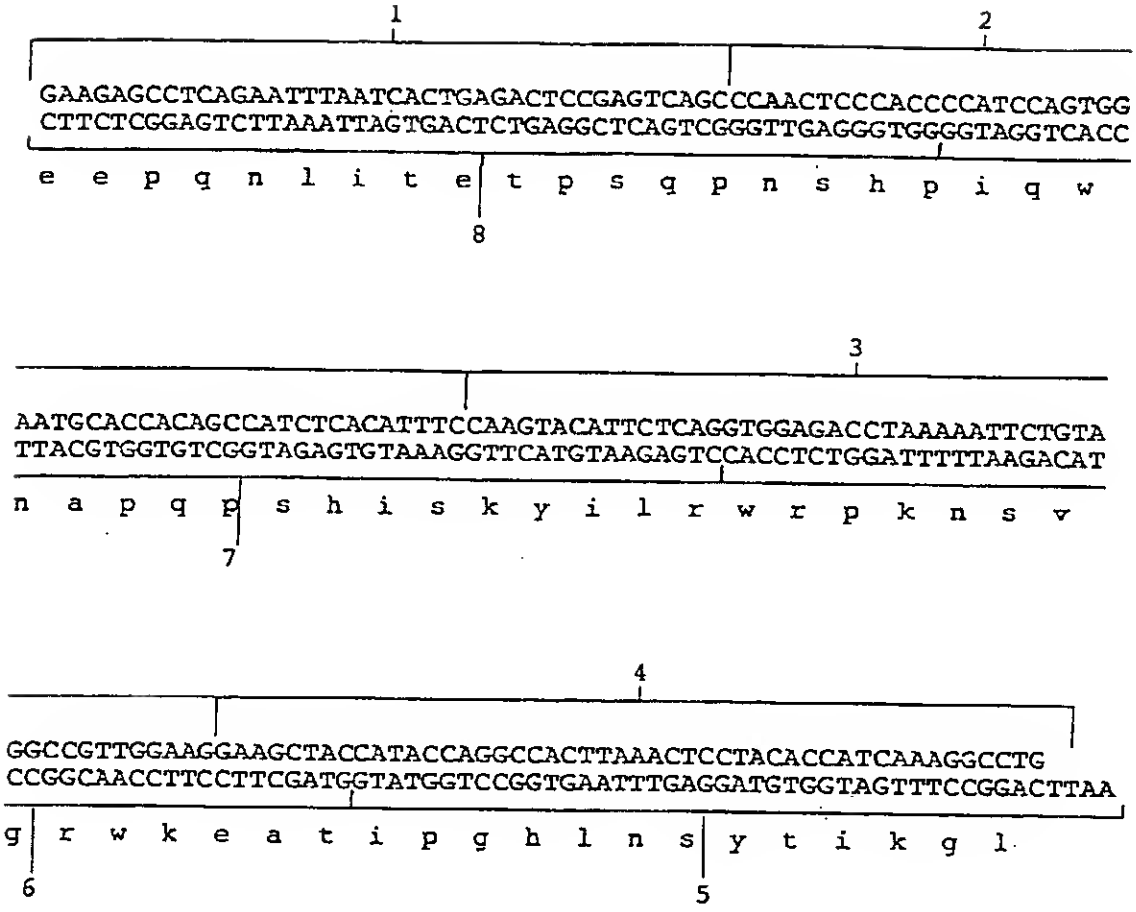


Figure 6 Linker 5 showing the eight constituent oligonucleotides

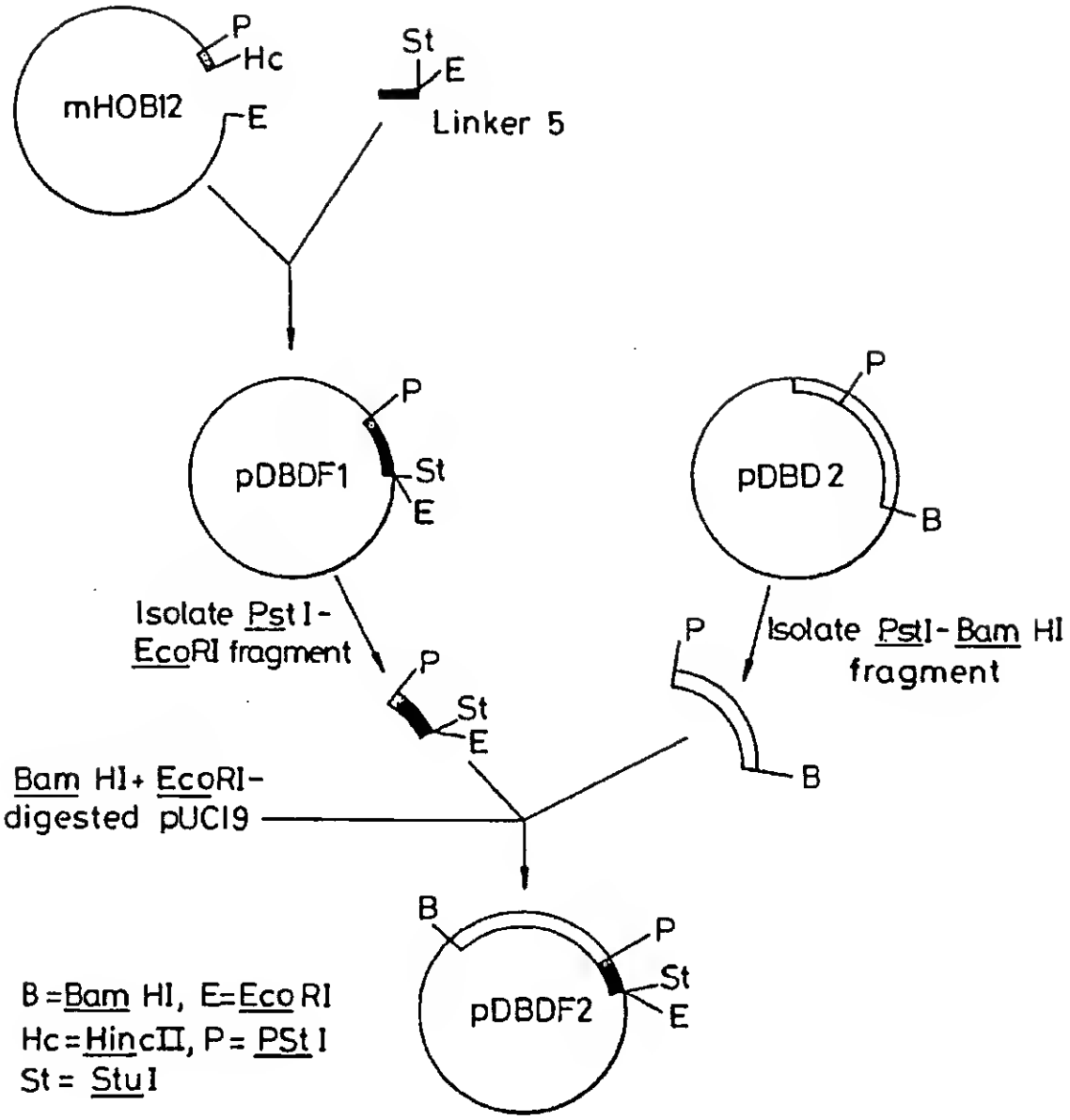


Fig. 7 Construction of pDBDF2

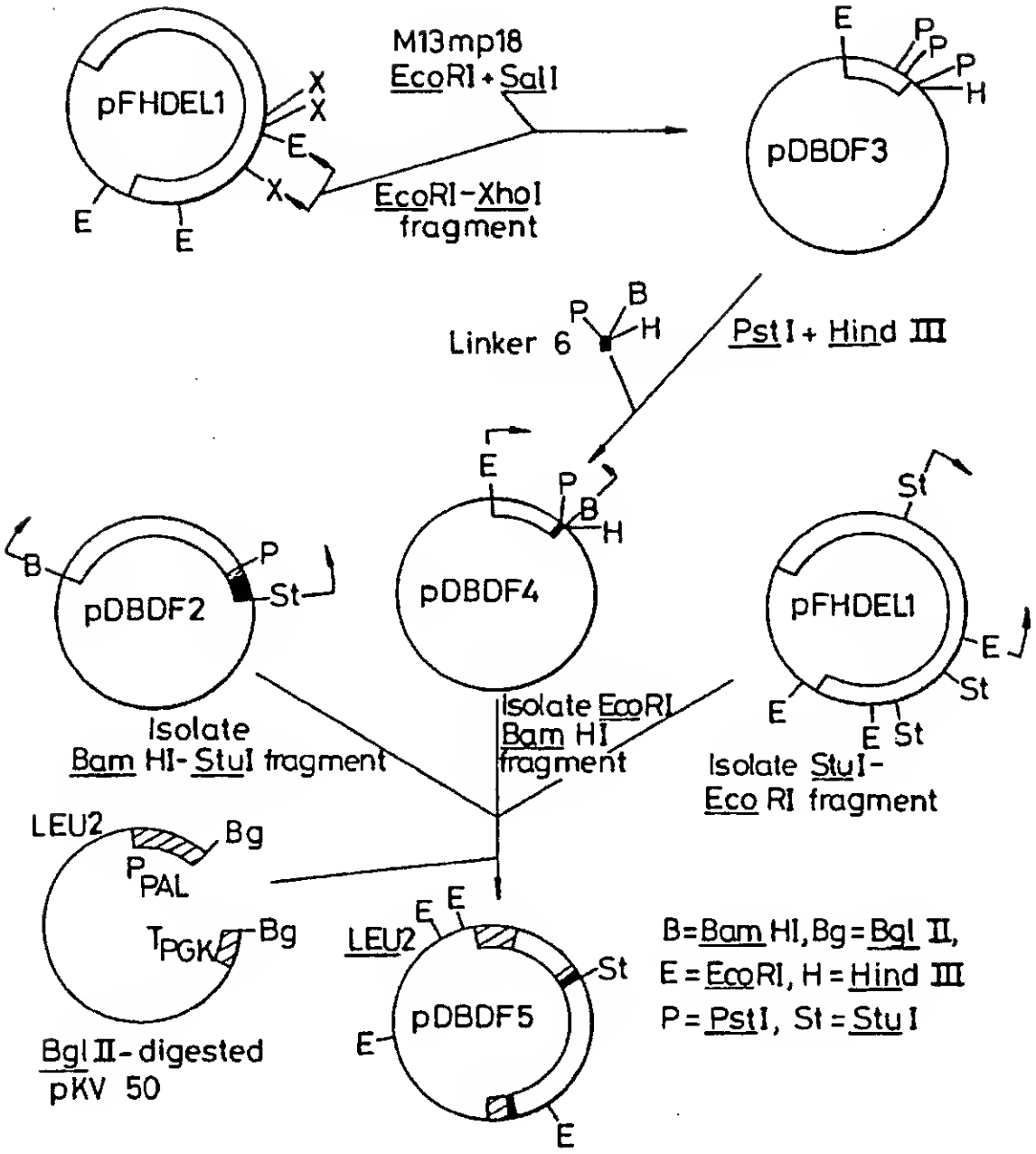


Fig. 8 Construction of pDBDF5

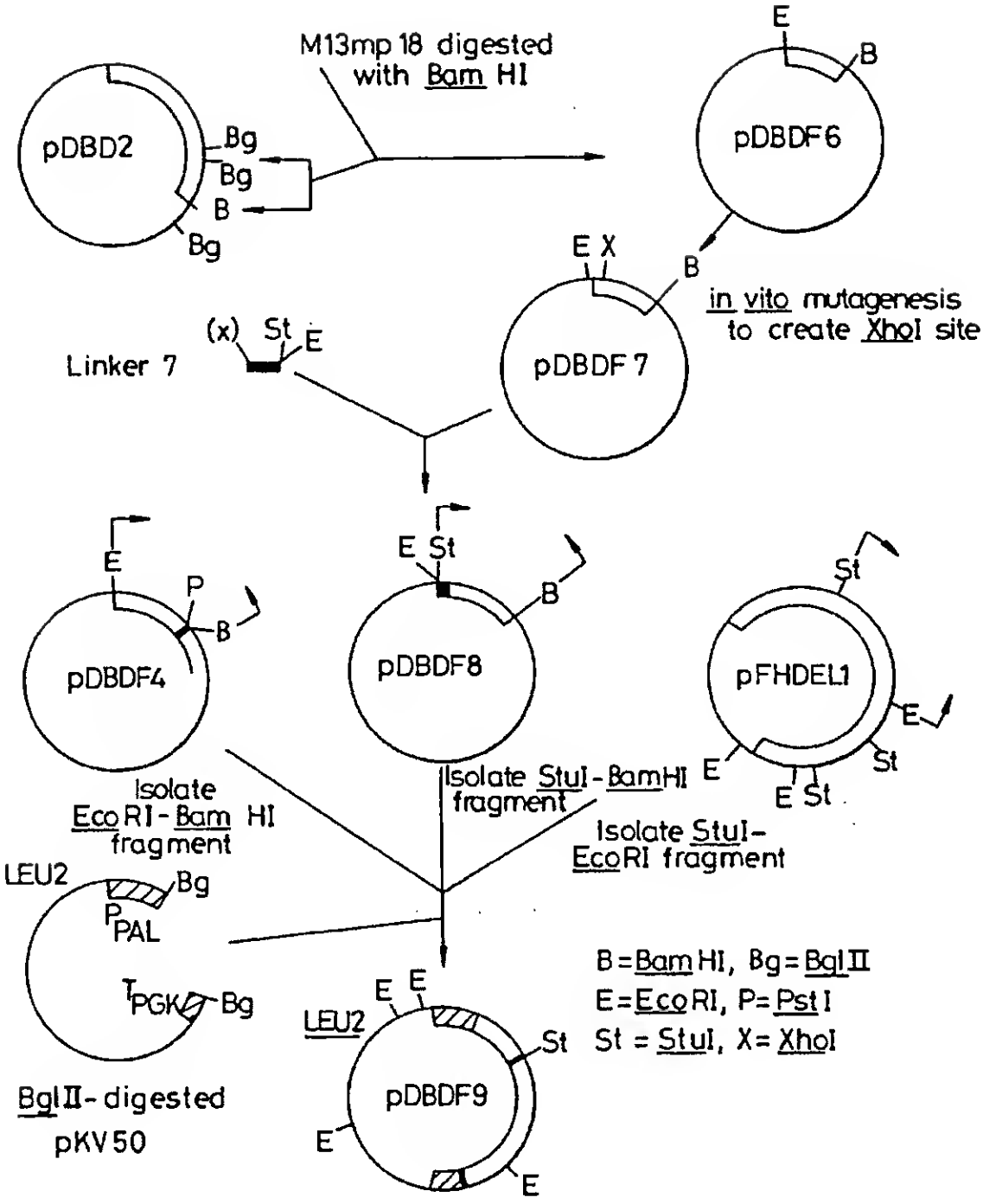


Fig. 9 Construction of pDBDF9

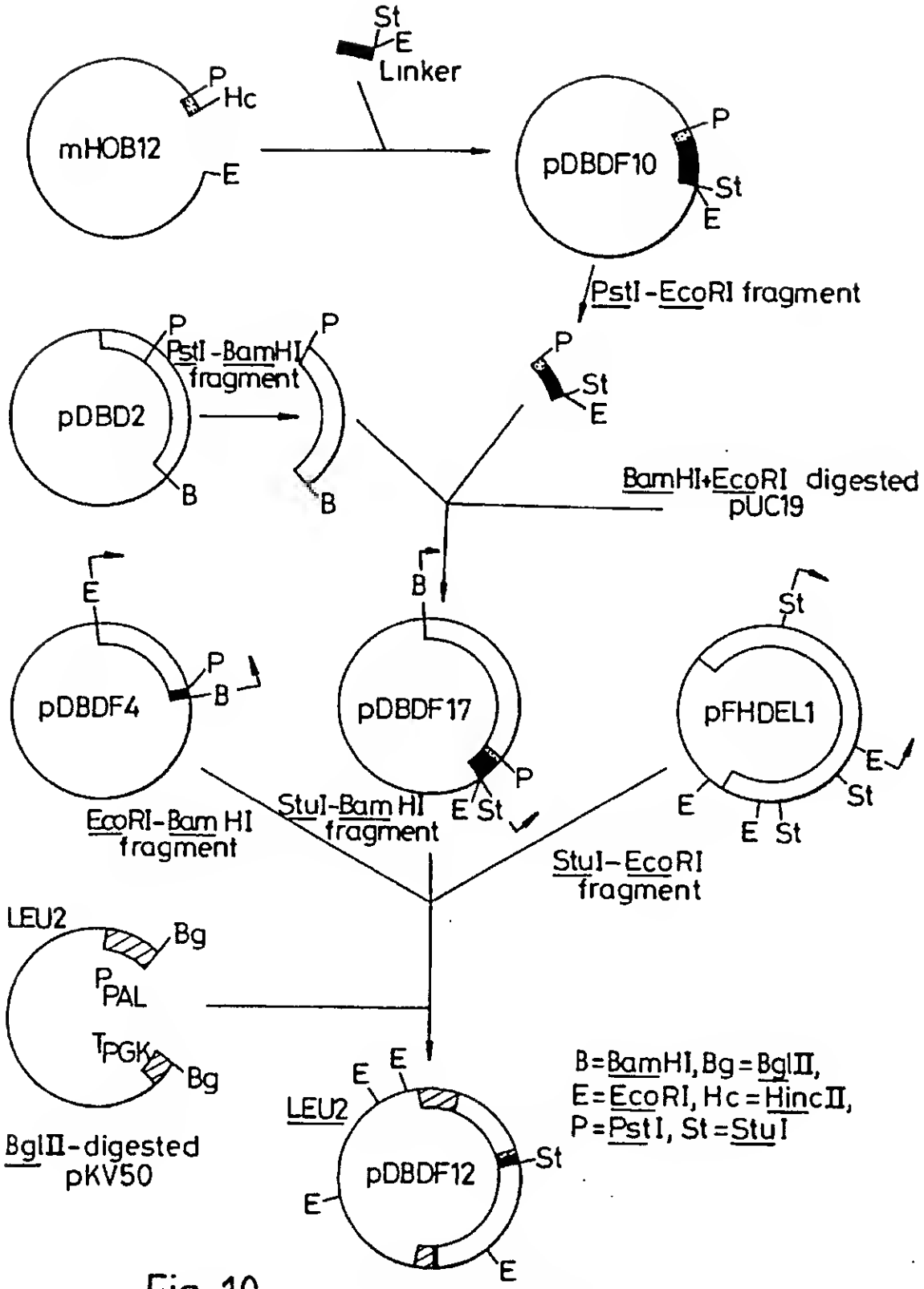


Fig. 10 Construction of pDBDF12

Figure 11

Name: pFHDEL1
Vector: pUC18 Amp^{r} 2860bp
Insert: hFNcDNA - 7630bp